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(54) Title: NUCLEIC ACIDS FOR INHIBITING HAIRLESS PROTEIN EXPRESSION AND METHODS OF USE THEREOF

(57) Abstract: The present invention provides DNAzymes and ribozymes that specifically cleave Hairless Protein mRNA. The present invention also provides antisense oligonucleotides that specifically inhibit translation of Hairless Protein mRNA. The invention also provides various methods of inhibiting the expression of Hairless Protein. Finally the invention provides pharmaceutical compositions containing the instant DNAzymes, ribozymes and antisense oligonucleotides as active ingredients.

NUCLEIC ACIDS FOR INHIBITING HAIRLESS PROTEIN EXPRESSION AND METHODS OF USE THEREOF

This application is a continuation-in-part and claims the benefit of copending U.S. Provisional Application No. 60/283,618, filed April 13, 2001, the contents of which are hereby incorporated by reference.

Background of the Invention

The Hairless Gene

The hairless mouse, a frequent subject of different dermatological experiments, is characterized by hair loss that starts at the age of 14 days postpartum from the upper eyelids and progresses caudally. The process is completed by the age of 3 weeks, when the mice are entirely naked and never grow hair again (Panteleyev et al. 1998b). The phenotype results from proviral integration and consequential aberrant splicing in the mouse hairless gene. Lack of expression of the mouse hairless gene due to inherited mutations leads to the complete loss of hair, known as atrichia. Other mutations have been identified in other mouse hairless alleles, and its human equivalent, resulting in essentially similar phenotypes (Ahmad et al. 1998a; Ahmad et al. 1998b; Panteleyev et al. 1998a). Those studies demonstrated that hairless expression in the hair follicle is necessary for hair cycling, specifically in the transition to the catagen phase.

Catalytic Nucleic Acid Molecules

Gene therapy is perhaps the most exciting promise of modern medical science. The technology of replacing mutant genes with correct ones can provide definitive therapy for a number of diseases. There are, however, conditions - inherited and acquired alike - which cannot be treated by the introduction of a new gene. In many cases, the ablation of an already existing gene may be desirable. In many dominantly inherited diseases, the successful "knock-out" of the mutant gene is, in theory, sufficient to cure the disease. In some other cases, the elimination of a normally functioning, wild-type gene may be necessary for therapeutic gene targeting.

Such is the case in abundant hair growth or hirsutism, in which inhibiting genes which promote hair growth could lead to decreased hair growth and, therefore, improvement. One way to achieve targeted, transient gene suppression is likely going to be through the use of catalytic nucleic acid technology, which includes both ribozymes and DNAzymes.

Ribozymes are RNA structures having a self-catalytic enzymatic function which, together with their sequence-specific and RNA-binding ability, make them capable of cleaving other RNA molecules at specific target sequences (Cech 1987). Recent success has been achieved in engineering ribozymes capable of selectively recognizing target sequences carrying different types of mutations, including single base-pair missense mutations (Parthasarathy et al. 1999; Sioud and Drlica 1991; Vaish et al. 1998).

These encouraging achievements give new perspective to

experimental strategies using selective mRNA ablation (Phylactou et al. 1998). The different groups of ribozymes described thus far (including hairpin ribozymes, hammerhead ribozymes and group I intron ribozymes (Bartel 1999)) have different characteristics with respect to their mechanism of splicing, splicing efficiency and target specificity. Several studies have used hammerhead ribozymes to selectively cleave RNA because of the superior target specificity of these ribozymes (Long and Sullenger 1999; Phylactou et al. 1998; Vaish et al. 1998).

Ribozymes can be delivered exogenously, such that the ribozymes are synthesized *in vitro*. They are usually administered using carrier molecules (Sioud 1996) or without carriers, using ribozymes specially modified to be nuclease-resistant (Flory et al. 1996). The other method is endogenous delivery, in which the ribozymes are inserted into a vector (usually a retroviral vector) which is then used to transfect target cells. There are several possible cassette constructs to chose from (Vaish et al. 1998), including the widely used U1 snRNA expression cassette, which proved to be efficient in nuclear expression of hammerhead ribozymes in various experiments (Bertrand et al. 1997; Michienzi et al. 1996; Montgomery and Dietz 1997).

Recent efforts have led to the successful development of small DNA oligonucleotides that have a structure similar to the hammerhead ribozyme (Santoro and Joyce 1997). These molecules are known as "deoxy-ribozymes", "deoxyribozymes" and "DNAzymes", and are virtually DNA equivalents of the hammerhead ribozymes. They consist of a 15 bp catalytic core and two sequence-specific arms with a typical length of 5-13 bp each (Santoro and Joyce

1998). Deoxy-ribozymes have more lenient consensus cleavage site requirements than hammerhead ribozymes, and are less likely to degrade when used for *in vivo* applications. The most widely used type of these novel catalytic molecules is known as the "10-23" deoxy-ribozyme, whose designation originates from the numbering used by its developers (Santoro and Joyce 1997). Because of their considerable advantages, deoxy-ribozymes have already been used in a wide spectrum of *in vitro* and *in vivo* applications (Cairns et al. 2000; Santiago et al. 1999).

Summary Of The Invention

This invention provides a catalytic deoxyribonucleic acid molecule that specifically cleaves Hairless Protein mRNA comprising:

(a) a catalytic domain that cleaves mRNA at a defined consensus sequence;

(b) a binding domain contiguous with the 5' end of the catalytic domain; and

(c) a binding domain contiguous with the 3' end of the catalytic domain,

wherein the binding domains are complementary to, and therefore hybridize with, the two regions flanking the defined consensus sequence within the Hairless Protein mRNA at which cleavage is desired, and wherein each binding domain is at least 4 residues in length, and both binding domains have a combined total length of at least 8 residues.

This invention also provides a catalytic ribonucleic acid molecule that specifically cleaves Hairless Protein mRNA comprising:

(a) a catalytic domain that cleaves mRNA at a defined consensus sequence;

(b) a binding domain contiguous with the 5' end of the catalytic domain; and

(c) a binding domain contiguous with the 3' end of the catalytic domain,

wherein the binding domains are complementary to, and therefore hybridize with, the two regions flanking the defined consensus sequence within the Hairless Protein mRNA at which cleavage is desired, and wherein each binding domain is at least 4 residues in length, and both binding domains have a combined total length of at least 8 residues.

This invention also provides a first pharmaceutical composition comprising the instant catalytic ribonucleic acid molecule or deoxyribonucleic acid molecule and a pharmaceutically acceptable carrier.

This invention further provides a method of specifically cleaving Hairless Protein mRNA comprising contacting the mRNA with either of the instant catalytic nucleic acid molecules under conditions permitting the molecule to cleave the mRNA.

This invention further provides a method of specifically cleaving Hairless Protein mRNA in a cell, comprising contacting the cell containing the mRNA with either of the instant catalytic nucleic acid molecules so as to specifically cleave the hairless protein mRNA in the cell.

This invention further provides a method of specifically inhibiting the expression of Hairless Protein in a cell that would otherwise express Hairless Protein, comprising contacting the cell with either of the instant catalytic nucleic acid molecules so as to specifically inhibit the expression of Hairless Protein in the cell.

This invention further provides a method of specifically inhibiting the expression of Hairless Protein in a subject's cells comprising administering to the subject an amount of either of the instant catalytic nucleic acid molecules effective to specifically inhibit the expression of Hairless Protein in the subject's cells.

This invention further provides a method of specifically inhibiting the expression of Hairless Protein in a subject's cells comprising administering to the subject

an amount of the first pharmaceutical composition effective to specifically inhibit the expression of Hairless Protein in the subject's cells.

This invention further provides a method of inhibiting hair production by a hair-producing cell comprising contacting the cell with an effective amount of either of the instant catalytic nucleic acid molecules.

This invention further provides a method of inhibiting hair growth in a subject comprising administering to the subject an effective amount of the first pharmaceutical composition.

This invention further provides a method of inhibiting the transition of a hair follicle from the anagen phase to the catagen phase comprising contacting the follicle with an effective amount of either of the instant catalytic nucleic acid molecules or the first pharmaceutical composition.

This invention further provides a vector which comprises a sequence encoding either of the instant catalytic nucleic acid molecules.

This invention further provides a host-vector system comprising a cell having the instant vector therein. This invention still further provides a method of producing either of the instant catalytic nucleic acid molecules comprising culturing a cell having therein a vector comprising a sequence encoding either catalytic nucleic acid molecule under conditions permitting the expression of the catalytic nucleic acid molecule by the cell.

This invention further provides a nucleic acid molecule

that specifically hybridizes to Hairless Protein mRNA so as to inhibit the translation thereof in a cell.

The invention further provides a second pharmaceutical composition comprising (a) the instant nucleic acid molecule or the instant vector and (b) a pharmaceutically acceptable carrier.

This invention further provides a method of specifically inhibiting the expression of Hairless Protein in a cell that would otherwise express Hairless Protein, comprising contacting the cell with the instant nucleic acid molecule so as to specifically inhibit the expression of Hairless Protein in the cell.

This invention further provides a method of specifically inhibiting the expression of Hairless Protein in a subject's cells comprising administering to the subject an amount of the instant nucleic acid molecule effective to specifically inhibit the expression of Hairless Protein in the subject's cells.

This invention further provides a method of specifically inhibiting the expression of Hairless Protein in a subject's cells comprising administering to the subject an amount of the second pharmaceutical composition effective to specifically inhibit the expression of Hairless Protein in the subject's cells.

This invention further provides a method of inhibiting hair production by a hair-producing cell comprising contacting the cell with an effective amount of the instant nucleic acid molecule.

This invention further provides a method of inhibiting

hair growth in a subject comprising administering to the subject an effective amount of the second pharmaceutical composition.

This invention further provides a method of inhibiting the transition of a hair follicle from the anagen phase to the catagen phase comprising contacting the follicle with an effective amount of the instant nucleic acid molecule or the second pharmaceutical composition.

This invention further provides a method of producing the instant nucleic acid molecule comprising culturing a cell having therein a vector comprising a sequence encoding said nucleic acid molecule under conditions permitting the expression of the nucleic acid molecule by the cell.

Finally this invention provides a non-human transgenic mammal, wherein the mammal's genome:

(a) has stably integrated therein a nucleotide sequence encoding a human Hairless Protein operably linked to a promoter, whereby the nucleotide sequence is expressed; and

(b) lacks an expressible endogenous hairless Protein-encoding nucleic acid sequence.

Brief Description of the Figures

Figures 1A - 1C: These figures show the mRNA sequence of Human Hairless Protein.

Figures 2A - 2D: Figures 2B - 2D show the pathology of C67BL/J mice treated with anti-Hairless Protein deoxyribozymes. Figure 2A shows a control area treated with a non-specific deoxyribozyme.

Figure 3: This figure shows reverse transcriptase polymerase chain reaction products visualized on an ethidium bromide-containing 2% agarose gel under UV light. Those deoxyribozymes that were capable of cleaving the target mouse Hairless Protein mRNA with the highest efficiency were used for *in vivo* experiments, as shown in lanes 1 ("702") and 3 ("754\sht").

Figure 4: This figure shows antisense oligonucleotide inhibition of Hairless Protein expression in Cos-1 cells. Lane 1 is the negative control where cells were not transfected with the full-length Hairless construct. Lane 2 shows Hairless expression in the positive control experiment where antisense oligonucleotides were not introduced. Lanes 3 and 4 show the inhibition of Hairless where anti-Hairless antisense ODN1 (SEQ ID NO:26) and ODN2 (SEQ ID NO:27), respectively, were added to the medium at 40 μM concentration prior to transfection.

Detailed Description of the Invention

Definitions

As used herein, and unless stated otherwise, each of the following terms shall have the definition set forth below.

"Administering" shall mean administering according to any of the various methods and delivery systems known to those skilled in the art. The administering can be performed, for example, via implant, transmucosally, transdermally and subcutaneously. In the preferred embodiment, the administering is topical and preferably dermal.

"Catalytic" shall mean the functioning of an agent as a catalyst, i.e. an agent that increases the rate of a chemical reaction without itself undergoing a permanent structural change.

"Consensus sequence" shall mean a nucleotide sequence of at least two residues in length between which catalytic nucleic acid cleavage occurs. For example, consensus sequences include "A:C" and "G:U".

"Hairless Protein" shall mean the protein encoded by the nucleotide sequence shown in Figures 1A - 1C (SEQ ID NO:1) and having the amino acid sequence shown in SEQ ID NO:17, and any variants thereof, whether artificial or naturally occurring. Variants include, without limitation, homologues, post-translational modifications, mutants such as those commonly referred to as T1022A, 1256delC, 1261del21, R620Q, 2001delCCAG, 2776+1G→A, N970K, V1136D, 3434delC and 2147delC, and polymorphisms

such as the one commonly referred to as L526P.

"Hairless Protein mRNA" shall mean any mRNA molecule comprising a sequence which encodes Hairless Protein. Hairless Protein mRNA includes, without limitation, protein-encoding sequences as well as the 5' and 3' non-protein-encoding sequences. An example of Hairless Protein mRNA is the mRNA sequence shown in Figure 1. As used herein, the terms "Hairless Protein", "Hairless", "hairless protein" and "hairless" are used interchangeably, unless stated otherwise.

"Hybridize" shall mean the annealing of one single-stranded nucleic acid molecule to another nucleic acid molecule based on sequence complementarity. The propensity for hybridization between nucleic acids depends on the temperature and ionic strength of their milieu, the length of the nucleic acids and the degree of complementarity. The effect of these parameters on hybridization is well known in the art (see Sambrook, 1989).

"Inhibit" shall mean to slow, stop or otherwise impede.

"Nucleic acid molecule" shall mean any nucleic acid molecule, including, without limitation, DNA, RNA and hybrids thereof. The nucleic acid bases that form nucleic acid molecules can be the bases A, C, G, T and U, as well as derivatives thereof. Derivatives of these bases are well known in the art, and are exemplified in PCR Systems, Reagents and Consumables (Perkin Elmer Catalogue 1996-1997, Roche Molecular Systems, Inc., Branchburg, New Jersey, USA).

"Pharmaceutically acceptable carrier" shall mean any of

the various carriers known to those skilled in the art. In one embodiment, the carrier is an alcohol, preferably ethylene glycol. In another embodiment, the carrier is a liposome. The following pharmaceutically acceptable carriers are set forth, in relation to their most commonly associated delivery systems, by way of example, noting the fact that the instant pharmaceutical compositions are preferably delivered dermally.

Dermal delivery systems include, for example, aqueous and nonaqueous gels, creams, multiple emulsions, microemulsions, liposomes, ointments, aqueous and nonaqueous solutions, lotions, aerosols, hydrocarbon bases and powders, and can contain excipients such as solubilizers, permeation enhancers (e.g., fatty acids, fatty acid esters, fatty alcohols and amino acids), and hydrophilic polymers (e.g., polycarbophil and polyvinylpyrolidone). In one embodiment, the pharmaceutically acceptable carrier is a liposome or a transdermal enhancer. Examples of liposomes which can be used in this invention include the following: (1) CellFectin, 1:1.5 (M/M) liposome formulation of the cationic lipid N,NI,NII,NIII-tetramethyl-N,NI,NII,NIII-tetrapalmity-spermine and dioleoyl phosphatidylethanol-amine (DOPE)(GIBCO BRL); (2) Cytofectin GSV, 2:1 (M/M) liposome formulation of a cationic lipid and DOPE (Glen Research); (3) DOTAP (N-[1-(2,3-dioleoyloxy)-N,N,N-tri-methyl-ammoniummethylsulfate) (Boehringer Manheim); and (4) Lipofectamine, 3:1 (M/M) liposome formulation of the polycationic lipid DOSPA and the neutral lipid DOPE (GIBCO BRL).

Transmucosal delivery systems include patches, tablets, suppositories, pessaries, gels and creams, and can contain excipients such as solubilizers and enhancers

(e.g., propylene glycol, bile salts and amino acids), and other vehicles (e.g., polyethylene glycol, fatty acid esters and derivatives, and hydrophilic polymers such as hydroxypropylmethylcellulose and hyaluronic acid).

Injectable drug delivery systems include solutions, suspensions, gels, microspheres and polymeric injectables, and can comprise excipients such as solubility-altering agents (e.g., ethanol, propylene glycol and sucrose) and polymers (e.g., polycaprylactones and PLGA's). Implantable systems include rods and discs, and can contain excipients such as PLGA and polycaprylactone.

Oral delivery systems include tablets and capsules. These can contain excipients such as binders (e.g., hydroxypropylmethylcellulose, polyvinyl pyrilodone, other cellulosic materials and starch), diluents (e.g., lactose and other sugars, starch, dicalcium phosphate and cellulosic materials), disintegrating agents (e.g., starch polymers and cellulosic materials) and lubricating agents (e.g., stearates and talc).

"Specifically cleave", when referring to the action of one of the instant catalytic nucleic acid molecules on a target mRNA molecule, shall mean to cleave the target mRNA molecule without cleaving another mRNA molecule lacking a sequence complementary to either of the catalytic nucleic acid molecule's two binding domains.

"Subject" shall mean any animal, such as a human, a primate, a mouse, a rat, a guinea pig or a rabbit.

"Vector" shall include, without limitation, a nucleic acid molecule that can be used to stably introduce a

specific nucleic acid sequence into the genome of an organism.

Finally, the following abbreviations shall have the meanings set forth below: "A" shall mean Adenine; "bp" shall mean base pairs; "C" shall mean Cytosine; "DNA" shall mean deoxyribonucleic acid; "G" shall mean Guanine; "mRNA" shall mean messenger ribonucleic acid; "RNA" shall mean ribonucleic acid; "RT-PCR" shall mean reverse transcriptase polymerase chain reaction; "RY" shall mean purine:pyrimidine; "T" shall mean Thymine; and "U" shall mean Uracil.

Embodiments of the Invention

This invention provides a catalytic deoxyribonucleic acid molecule that specifically cleaves Hairless Protein mRNA comprising:

(a) a catalytic domain that cleaves mRNA at a defined consensus sequence;

(b) a binding domain contiguous with the 5' end of the catalytic domain; and

(c) a binding domain contiguous with the 3' end of the catalytic domain,

wherein the binding domains are complementary to, and therefore hybridize with, the two regions flanking the defined consensus sequence within the Hairless Protein mRNA at which cleavage is desired, and wherein each binding domain is at least 4 residues in length, and both binding domains have a combined total length of at least 8 residues. In the preferred embodiment, each binding domain is 7 residues in length, and both binding domains have a combined total length of 14 residues.

The catalytic domain may optionally contain stem-loop

structures in addition to the nucleotides required for catalytic activity. In one embodiment of the instant catalytic deoxyribonucleic acid molecule, the catalytic domain has the sequence ggctagctacaacga (SEQ ID NO:18), and cleaves mRNA at the consensus sequence purine:pyrimidine. In a preferred embodiment, cleavage occurs at one or more of the following cleavage sites in the Hairless Protein mRNA (shown in Figures 1A - 1C and SEQ ID NO:1), wherein the indicated nucleotide residue immediately precedes the cleavage site: 1594, 1597, 1641, 1698, 1732, 1750, 1801, 1811, 2028, 2033, 2047, 2083, 2269, 2380 and 2395.

In another embodiment the instant deoxyribonucleic acid molecule has a sequence selected from the group consisting of:

(a) cccatggggctagctacaacgagcagtcc (SEQ ID NO:2);
(b) cggcccaggctagctacaacgaggtgcag (SEQ ID NO:3);
(c) ctcaggaggctagctacaacgagcccctc (SEQ ID NO:4);
(d) gggagcaggctagctacaacgagtccttg (SEQ ID NO:5);
(e) ctccccaggctagctacaacgatctgggg (SEQ ID NO:6);
(f) cagccagggctagctacaacgatgacctt (SEQ ID NO:7);
(g) cagcggaggctagctacaacgagggtaag (SEQ ID NO:8);
(h) cgcagaaggctagctacaacgagccagcg (SEQ ID NO:9);
(i) tgcgggggctagctacaacgacaggggc (SEQ ID NO:10);
(j) aggggtgggctagctacaacgaggggtca (SEQ ID NO:11);
(k) cccggagggctagctacaacgaataccca (SEQ ID NO:12);
(l) gcctaagggctagctacaacgatgaaggc (SEQ ID NO:13);
(m) gcaaggaggctagctacaacgatctgctg (SEQ ID NO:14);
(n) gttcccaggctagctacaacgacgcctgg (SEQ ID NO:15); and
(o) cagctggggctagctacaacgaacccaag (SEQ ID NO:16).

This invention also provides a catalytic ribonucleic acid molecule that specifically cleaves Hairless Protein mRNA

comprising:

(a) a catalytic domain that cleaves mRNA at a defined consensus sequence;

(b) a binding domain contiguous with the 5' end of the catalytic domain; and

(c) a binding domain contiguous with the 3' end of the catalytic domain,

wherein the binding domains are complementary to, and therefore hybridize with, the two regions flanking the defined consensus sequence within the Hairless Protein mRNA at which cleavage is desired, and wherein each binding domain is at least 4 residues in length and both binding domains have a combined total length of at least 8 residues.

In one embodiment of the instant catalytic ribonucleic acid molecule, each binding domain is at least 12 residues in length. In the preferred embodiment, each binding domain is no more than 17 residues in length. In another embodiment, both binding domains have a combined total length of at least 24 residues, and no more than 34 residues.

Preferably, the instant catalytic ribonucleic acid molecule is a hammerhead ribozyme. Hammerhead ribozymes are well known in the literature, as described in Pley et al, 1994. In one embodiment, the consensus sequence is the sequence 5'-NUH-3', where N is any nucleotide, U is uridine and H is any nucleotide except guanine. An example of such sequence is 5'-adenine:uracil:adenine-3'. In another embodiment, the catalytic domain has the sequence ctgatgagtccgtgaggacgaaaca (SEQ ID NO:19).

In this invention, the instant catalytic nucleic acid molecules can cleave Hairless Protein mRNA at each and

any of the consensus sequences therein. Since ribozyme and DNAzyme consensus sequences are known, and the Hairless Protein mRNA sequence is known, one of ordinary skill could readily construct a catalytic nucleic acid molecule directed to any of the Hairless Protein mRNA consensus sequences based on the instant specification. In a preferred embodiment, the cleavage occurs at one or more of the following cleavage sites in Hairless Protein mRNA (sequence shown in Figures 1A - 1C and SEQ ID NO:1), wherein the indicated nucleotide residue immediately precedes the cleavage site: -94, 159, 264, 506, 847 and 879. The number -94 indicates a target site upstream from the 5' end of the sequence shown in Figure 1, in the 5' untranslated region of the sequence.

In another embodiment, the instant catalytic ribonucleic acid molecule has a sequence selected from the group consisting of:

(a) cggccggcgggcgagctgatgagtccgtgaggacgaaacacgcgttctcc cgctct (SEQ ID NO:20);

(b) gagtctggggtgctcagctgatgagtccgtgaggacgaaacacgcccctc caaaaagg (SEQ ID NO:21);

(c) ttgctgcccagccagttctgatgagtccgtgaggacgaaacaccttcctc tccccatt (SEQ ID NO:22);

(d) gctctgggggcaggccactgatgagtccgtgaggacgaaacacactaggt agggtggc (SEQ ID NO:23);

(e) atgaacaaggcctggggctgatgagtccgtgaggacgaaacacaagcggg ccaggagg (SEQ ID NO:24); and

(f) tcgcctggcccagcccactgatgagtccgtgaggacgaaacacgttgcca agagtatg (SEQ ID NO:25).

In an alternative embodiment of the instant catalytic ribonucleic acid molecule, the molecule is a hairpin ribozyme. Hairpin ribozymes are well known in the literature as described in Fedor (2000).

Catalytic nucleic acid molecules can be directed to any cleavage site within the Hairless Protein mRNA, preferably within the 5' half of the mRNA. In one embodiment the cleavage site within the hairless protein mRNA is located within the first 3000 residues following the mRNA's 5' terminus. In another embodiment cleavage occurs within the first 1500 residues. Here, "following" means in the 3' direction of the 5' terminus.

The Hairless Protein mRNA cleaved by the instant catalytic nucleic acid molecules can be from any subject. In one embodiment, the Hairless Protein mRNA is from a subject selected from the group consisting of human, monkey, rat and mouse, and in the preferred embodiment is human. In the preferred embodiment, the Hairless Protein mRNA has the sequence shown in Figures 1A - 1C.

This invention also provides a first pharmaceutical composition comprising the instant catalytic ribonucleic acid molecule or deoxyribonucleic acid molecule and a pharmaceutically acceptable carrier.

This invention further provides a method of specifically cleaving Hairless Protein mRNA comprising contacting the mRNA with either of the instant catalytic nucleic acid molecules under conditions permitting the molecule to cleave the mRNA. These conditions are well known in the art and include physiological conditions.

This invention further provides a method of specifically cleaving Hairless Protein mRNA in a cell, comprising contacting the cell containing the mRNA with either of the instant catalytic nucleic acid molecules so as to specifically cleave the hairless protein mRNA in the

cell. The cell containing Hairless Protein mRNA can be, for example, a naturally occurring cell or a transgenic cell. In the preferred embodiment, the cell is a keratinocyte.

This invention further provides a method of specifically inhibiting the expression of Hairless Protein in a cell that would otherwise express Hairless Protein, comprising contacting the cell with either of the instant catalytic nucleic acid molecules so as to specifically inhibit the expression of Hairless Protein in the cell.

This invention further provides a method of specifically inhibiting the expression of Hairless Protein in a subject's cells comprising administering to the subject an amount of either of the instant catalytic nucleic acid molecules effective to specifically inhibit the expression of Hairless Protein in the subject's cells.

This invention further provides a method of specifically inhibiting the expression of Hairless Protein in a subject's cells comprising administering to the subject an amount of the first pharmaceutical composition effective to specifically inhibit the expression of Hairless Protein in the subject's cells.

Determining the effective amount of the instant pharmaceutical composition can be done based on animal data using routine computational methods. In one embodiment, the effective amount contains between about 10 ng and about 100 μg of the instant nucleic acid molecules per quare centimeter of skin. In another embodiment, the effective amount contains between about 100 ng and about 10 μg of the nucleic acid molecules per square centimeter of skin. In a further embodiment, the

effective amount contains between about 1 μg and about 5 μg, and preferably about 2 μg, of the nucleic acid molecules per square centimeter of skin.

This invention further provides a method of inhibiting hair production by a hair-producing cell comprising contacting the cell with an effective amount of either of the instant catalytic nucleic acid molecules.

This invention further provides a method of inhibiting hair growth in a subject comprising administering to the subject an effective amount of the first pharmaceutical composition.

Hair follicles are dynamic structures that generate hair through a regulated cycle of growth and remodeling. The hair follicle cycles between rest (telogen), growth (anagen) and regression (catagen). This invention further provides a method of inhibiting the transition of a hair follicle from the anagen phase to the catagen phase comprising contacting the follicle with an effective amount of either of the instant catalytic nucleic acid molecules or the first pharmaceutical composition.

This invention further provides a vector which comprises a sequence encoding either of the instant catalytic nucleic acid molecules.

This invention further provides a host-vector system comprising a cell having the instant vector therein. This invention still further provides a method of producing either of the instant catalytic nucleic acid molecules comprising culturing a cell having therein a vector comprising a sequence encoding either catalytic nucleic acid molecule under conditions permitting the expression

of the catalytic nucleic acid molecule by the cell. Methods of culturing cells in order to permit expression and conditions permitting expression are well known in the art. For example see Sambrook et al. (1989). Such methods can optionally comprise a further step of recovering the nucleic acid product.

This invention provides a nucleic acid molecule that specifically hybridizes to Hairless Protein mRNA so as to inhibit the translation thereof in a cell.

In one embodiment, the instant nucleic acid is a ribonucleic acid. In another embodiment the nucleic acid is deoxyribonucleic acid.

The invention further provides a second pharmaceutical composition comprising (a) the instant nucleic acid molecule or the instant vector and (b) a pharmaceutically acceptable carrier.

This invention further provides a method of specifically inhibiting the expression of Hairless Protein in a cell that would otherwise express Hairless Protein, comprising contacting the cell with the instant nucleic acid molecule so as to specifically inhibit the expression of Hairless Protein in the cell.

This invention further provides a method of specifically inhibiting the expression of Hairless Protein in a subject's cells comprising administering to the subject an amount of the instant nucleic acid molecule effective to specifically inhibit the expression of Hairless Protein in the subject's cells.

This invention further provides a method of specifically

inhibiting the expression of Hairless Protein in a subject's cells comprising administering to the subject an amount of the second pharmaceutical composition effective to specifically inhibit the expression of Hairless Protein in the subject's cells.

This invention further provides a method of inhibiting hair production by a hair-producing cell comprising contacting the cell with an effective amount of the instant nucleic acid molecule.

This invention further provides a method of inhibiting hair growth in a subject comprising administering to the subject an effective amount of the second pharmaceutical composition.

This invention further provides a method of inhibiting the transition of a hair follicle from the anagen phase to the catagen phase comprising contacting the follicle with an effective amount of the instant nucleic acid molecule or the second pharmaceutical composition.

This invention further provides a method of producing the instant nucleic acid molecule comprising culturing a cell having therein a vector comprising a sequence encoding said nucleic acid molecule under conditions permitting the expression of the nucleic acid molecule by the cell.

Finally this invention provides a non-human transgenic mammal, wherein the mammal's genome:

(a) has stably integrated therein a nucleotide sequence encoding a human Hairless Protein operably linked to a promoter, whereby the nucleotide sequence is expressed; and

(b) lacks an expressible endogenous Hairless Protein-

encoding nucleic acid sequence.

In the preferred embodiment, the transgenic mammal is a mouse. The instant transgenic mammal is useful as a model for testing hair removal products which function by inhibiting Hairless Protein expression.

In this invention, the various embodiments of subjects, pharmaceutically acceptable carriers, dosages, cell types, routes of administration and target nucleic acid sequences are envisioned for each of the instant nucleic acid molecules, pharmaceutical compositions and methods. Moreover, in this invention, the various embodiments of methods, subjects, pharmaceutically acceptable carriers, dosages, cell types, routes of administration and target nucleic acid sequences are envisioned for all non-nucleic acid agents which inhibit the expression of Hairless Protein. Such non-nucleic acid agents include, without limitation, polypeptides, carbohydrates and small organic compounds.

This invention will be better understood by reference to the Experimental Details which follow, but those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative of the invention as described more fully in the claims which follow thereafter.

Experimental Details

Part I - Catalytic Nucleic Acids

Introduction

Catalytic nucleic acid technology is widely used to target mRNA in a sequence-specific fashion, and thus change the expression pattern of cells or tissues. While the goal of mRNA targeting is usually the cleavage of mutant mRNA with the prospect of gene therapy for inherited diseases, in certain instances targeting of wild-type genes can be used therapeutically.

Lack of expression of the mouse hairless gene due to inherited mutations leads to the complete loss of hair, known as atrichia. This study was designed to recapitulate the hairless phenotype in a restricted manner by topical application of deoxy-ribozyme molecules to specifically cleave the mouse hairless mRNA. Pathology samples taken from the treated area at different times demonstrated a decreased number of hair follicles, involution of the remaining follicles, separation of the dermal papillae and the presence of dermal cysts, all characteristics of the hairless phenotype but not normally present in the skin of C57Bl/6J mice.

In this study, the hairless phenotype is successfully recapitulated using topically applied target-specific catalytic oligonucleotides designed to cleave the mouse hairless mRNA. Hence, this invention demonstrates the feasibility of using ribozyme and deoxy-ribozyme technology to alter gene expression in the skin via topical application and provide permanent hair removal.

Materials and Methods

Deoxy-ribozyme design and in vitro testing. To target the mouse hairless mRNA, a series of deoxy-ribozymes were designed based on the consensus cleavage sites 5'-RY-3' in the mRNA sequence (GenBank Accession#: AF039196) (Ahmad et al. 1998a). Only those potential cleavage sites which were located on an open loop of the mRNA according to the RNA folding software RNADRaw 2.1 were targeted (Matzura and Wennborg 1996). The deoxy-ribozyme design utilized the previously described structure (Santoro and Joyce 1997; Santoro and Joyce 1998) where two sequence-specific arms were attached to a catalytic core based on the mouse hairless mRNA sequence. The deoxy-ribozymes were custom synthesized (Life Technologies). Commercially available mouse brain polyA-RNA (Ambion) served as a template in the *in vitro* cleavage reaction to test the efficiency of the deoxy-ribozymes. 800 ng RNA template were incubated in the presence of 20mM Mg^{2+} and RNAse Out RNAse inhibitor (Life Technologies) at pH 7.5 with 2 μg deoxy-ribozyme for one hour. After incubation, aliquots of the reaction were used as templates for RT-PCR, amplifying regions including the targeted cleavage sites. The RT-PCR products were visualized on an ethidium bromide-containing 2% agarose gel under UV light, and the intensity of the products was determined as described above (Figure 3). Those deoxy-ribozymes that were capable of cleaving the target mouse hairless mRNA with the highest efficiency were used for *in vivo* experiments (Figure 3, lanes 1, 3).

Deoxy-ribozyme treatment schedule. Newborn C57Bl/6J mice were treated with a deoxy-ribozyme formula twice a day starting on the first day after delivery. As the mice

started to grow hair, hair shafts were regularly shortened using an electric clipper to make the skin surface accessible and to enhance the penetration of the treatment formula. For each treatment, 2 μg deoxy-ribozyme, dissolved in a 85% EtOH and 15% ethylene glycol vehicle, were applied to a one square centimeter area on the back. During application and for a fifteen minute period after, the mice were placed in temporary restraint to prevent removal of the formula. Control animals were treated with vehicle containing oligonucleotides of the same length but of random sequence. The treatment continued until the mice were sacrificed for evaluation.

Biopsy Procedures and Pathology. The mice were humanely euthanized after 28 days, 35 days or 8 weeks of treatment. The entire treatment area, together with an equal sized non-treated neighboring area of skin, were removed, fixed in formalin solution, embedded and processed for pathology using standard procedures.

Results

To evaluate the feasibility of using topically applied deoxy-ribozymes for selective ablation of genes expressed in the hair follicle, a model system was used to recapitulate the hairless mouse phenotype. After secondary structure based target-site selection, the choices were narrowed for targeting oligonucleotides using a novel *in vitro* cleavage assay (Cserhalmi-Friedman et al. manuscript in preparation). The three deoxy-ribozymes that proved to be the most efficient in cutting full-length mouse hairless mRNA *in vitro* were used for the *in vivo* experiments.

After continuous treatment, by day 20 the hair of the

treated animals became visibly sparse on the treated area. Pathology specimens taken from the treated area at day 28 (see Figures 2A - 2D) demonstrated (i) a decreased number of hair follicles, (ii) several dense, basophilic cell groups in the dermis corresponding to dermal papillae by morphology and localization, and (iii) an absence of surrounding epithelial hair follicle tissues and related hair follicles. The remaining hair follicles were in telogen phase, in sharp contrast with the advanced anagen follicles of the surrounding untreated skin and the skin of the untreated control animals.

Samples taken from the treated area at day 35 showed a different result. Some follicles in telogen phase could be observed, although they were more sparse than in the surrounding untreated area or in the samples from the untreated control animals. In the dermis of the treated region, large epithelial cysts filled with amorphous material were noticed, which corresponded to dermal cysts. These characteristics, the arrest of hair cycling, involution of hair follicles, detachment of dermal papillae and development of dermal cysts are not normally present in C57Bl/6J mice, but represent the cardinal features of the skin of the hairless mouse.

The data revealed here demonstrate that by using topically applied catalytic oligonucleotides, a key player of hair follicle regulation can be eliminated and hair follicle cycling can be disrupted. These results serve not only as a proof of principle for future use of this approach for hair removal, but also demonstrate the feasibility of using topical catalytic nucleic acid technology to successfully change the gene expression pattern of hair follicle cells at the mRNA level, and thus influence the hair phenotype.

Part II - Antisense Nucleic Acids

Antisense oligodeoxynucleotides were synthesized as directed to the inhibition of Hairless expression based on the Hairless mRNA sequence (SEQ ID NO:1). Cos-1 cells were transfected with the pEGFP full-length Hairless construct, and cells were harvested 16 hours after transfection. Total protein lysate was analyzed on a 10% SDS PAGE gel and transferred to nitrocellulose. Hairless protein was detected using an anti-Hairless polyclonal antibody raised against a C-terminal peptide. In a negative control, cells not transfected with the full-length Hairless construct showed no Hairless expression. Hairless expression was clearly observed in positive control experiments when antisense oligodeoxynucleotides were not added. Experiments where anti-Hairless antisense oligonucleotides (i.e. ODN1: 5' GCTGGGCATACTCTCCAT 3' (SEQ ID NO:26) and ODN2: 5' CATCACTCTCCTGCCCTC 3' (SEQ ID NO:27)) were added to the medium at 40 μM concentration prior to the transfection clearly showed an absence of Hairless expression, demonstrating antisense oligodeoxynucleotide inhibition of the Hairless gene product (see Figure 4).

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What is claimed is:

1. A catalytic deoxyribonucleic acid molecule that specifically cleaves Hairless Protein mRNA comprising:

(a) a catalytic domain that cleaves mRNA at a defined consensus sequence;

(b) a binding domain contiguous with the 5' end of the catalytic domain; and

(c) a binding domain contiguous with the 3' end of the catalytic domain,

wherein the binding domains are complementary to, and therefore hybridize with, the two regions flanking the defined consensus sequence within the Hairless Protein mRNA at which cleavage is desired, and wherein each binding domain is at least 4 residues in length and both binding domains have a combined total length of at least 8 residues.

2. The catalytic deoxyribonucleic acid molecule of claim 1, wherein the catalytic domain has the sequence ggctagctacaacga (SEQ ID NO:18), and cleaves mRNA at the consensus sequence purine:pyrimidine.

3. The catalytic deoxyribonucleic acid molecule of claim 2, wherein the molecule has a sequence selected from the group consisting of:

(a) cccatggggctagctacaacgagcagtcc (SEQ ID NO:2);

(b) cggcccaggctagctacaacgaggtgcag (SEQ ID NO:3);

(c) ctcaggaggctagctacaacgagcccctc (SEQ ID NO:4);

(d) gggagcaggctagctacaacgagtccttg (SEQ ID NO:5);

(e) ctccccaggctagctacaacgatctgggg (SEQ ID NO:6);

(f) cagccagggctagctacaacgatgacctt (SEQ ID NO:7);

(g) cagcggaggctagctacaacgagggtaag (SEQ ID NO:8);

(h) cgcagaaggctagctacaacgagccagcg (SEQ ID NO:9);
(i) tgcgggggctagctacaacgacaggggc (SEQ ID NO:10);
(j) aggggtgggctagctacaacgaggggtca (SEQ ID NO:11);
(k) cccggagggctagctacaacgaataccca (SEQ ID NO:12);
(l) gcctaagggctagctacaacgatgaaggc (SEQ ID NO:13);
(m) gcaaggaggctagctacaacgatctgctg (SEQ ID NO:14);
(n) gttcccaggctagctacaacgacgcctgg (SEQ ID NO:15); and
(o) cagctggggctagctacaacgaacccaag (SEQ ID NO:16).

4. A catalytic ribonucleic acid molecule that specifically cleaves Hairless Protein mRNA comprising:
(a) a catalytic domain that cleaves mRNA at a defined consensus sequence;
(b) a binding domain contiguous with the 5' end of the catalytic domain; and
(c) a binding domain contiguous with the 3' end of the catalytic domain,
wherein the binding domains are complementary to, and therefore hybridize with, the two regions flanking the defined consensus sequence within the Hairless Protein mRNA at which cleavage is desired, and wherein each binding domain is at least 4 residues in length and both binding domains have a combined total length of at least 8 residues.

5. The catalytic ribonucleic acid molecule of claim 4, wherein the molecule is a hammerhead ribozyme.

6. The catalytic ribonucleic acid molecule of claim 4, wherein the catalytic domain has the sequence ctgatgagtccgtgaggacgaaaca (SEQ ID NO:19), and cleaves mRNA at the consensus sequence 5'-NUH-3'.

7. The catalytic ribonucleic acid molecule of claim 6, wherein the catalytic ribonucleic acid molecule has a sequence selected from the group consisting of:

(a) cggccggcgggcgagctgatgagtccgtgaggacgaaaca cgcgttctcccgctct (SEQ ID NO:20);

(b) gagtctggggtgctcagctgatgagtccgtgaggacgaaa cacgcccctccaaaaagg (SEQ ID NO:21);

(c) ttgctgcccagccagttctgatgagtccgtgaggacgaaa caccttcctctccccatt (SEQ ID NO:22);

(d) gctctgggggcaggccactgatgagtccgtgaggacgaaa cacactaggtagggtggc (SEQ ID NO:23);

(e) atgaacaaggcctggggctgatgagtccgtgaggacgaaa cacaagcgggccaggagg (SEQ ID NO:24); and

(f) tcgcctggcccagcccactgatgagtccgtgaggacgaaa cacgttgccaagagtatg (SEQ ID NO:25).

8. The catalytic ribonucleic acid molecule of claim 4, wherein the molecule is a hairpin ribozyme.

9. The catalytic nucleic acid molecule of claim 1 or 4, wherein the cleavage site within the Hairless Protein mRNA is located within the first 3000 residues following the mRNA's 5' terminus.

10. The catalytic nucleic acid molecule of claim 9, wherein the cleavage site within the Hairless Protein mRNA is located within the first 1500 residues following the mRNA's 5' terminus.

11. The catalytic nucleic acid molecule of claim 1 or 4, wherein the Hairless Protein mRNA is from a subject selected from the group consisting of human, monkey, rat and mouse.

12. The catalytic nucleic acid molecule of claim 1 or 4, wherein the Hairless Protein mRNA has the sequence shown in Figures 1A - 1C (SEQ ID NO:1).

13. A pharmaceutical composition comprising the catalytic nucleic acid molecule of claim 1 or 4 and a pharmaceutically acceptable carrier.

14. The pharmaceutical composition of claim 13, wherein the carrier is an alcohol.

15. The pharmaceutical composition of claim 14, wherein the carrier is ethylene glycol.

16. The pharmaceutical composition of claim 13, wherein the carrier is a liposome.

17. A method of specifically cleaving Hairless Protein mRNA comprising contacting the mRNA with the catalytic nucleic acid molecule of claim 1 or 4 under conditions permitting the molecule to cleave the mRNA.

18. A method of specifically cleaving Hairless Protein mRNA in a cell, comprising contacting the cell containing the mRNA with the catalytic nucleic acid molecule of claim 1 or 4 so as to specifically cleave the Hairless Protein mRNA in the cell.

19. A method of specifically inhibiting the expression of Hairless Protein in a cell that would otherwise express Hairless Protein, comprising contacting the cell with the catalytic nucleic acid molecule of claim 1 or 4 so as to specifically inhibit the expression of Hairless Protein in the cell.

20. A method of specifically inhibiting the expression of Hairless Protein in a subject's cells comprising administering to the subject an amount of the catalytic nucleic acid molecule of claim 1 or 4 effective to specifically inhibit the expression of Hairless Protein in the subject's cells.

21. A method of specifically inhibiting the expression of Hairless Protein in a subject's cells comprising administering to the subject an amount of the pharmaceutical composition of claim 13 effective to specifically inhibit the expression of Hairless Protein in the subject's cells.

22. A method of inhibiting hair production by a hair-producing cell comprising contacting the cell with an effective amount of the catalytic nucleic acid of claim 1 or 4.

23. A method of inhibiting hair growth in a subject comprising administering to the subject an effective amount of the pharmaceutical composition of claim 13.

24. A method of inhibiting the transition of a hair follicle from the anagen phase to the catagen phase comprising contacting the follicle with an effective amount of the catalytic nucleic acid of claim 1 or 4.

25. A method of inhibiting the transition of a hair follicle from the anagen phase to the catagen phase comprising contacting the follicle with an effective amount of the pharmaceutical composition of claim 13.

26. The method of claim 18, wherein the cell is a keratinocyte.

27. The method of claim 19, wherein the cell is a keratinocyte.

28. The method of claim 20, wherein the cell is a keratinocyte.

29. The method of claim 21, wherein the cell is a keratinocyte.

30. The method of claim 22, wherein the cell is a keratinocyte.

31. The method of claim 20, wherein the subject is a human.

32. The method of claim 21, wherein the subject is a human.

33. The method of claim 23, wherein the subject is a human.

34. The method of claim 20, wherein the catalytic nucleic acid molecule is administered topically.

35. The method of claim 34, wherein the catalytic nucleic acid is administered dermally.

36. The method of claim 21, wherein the pharmaceutical composition is administered topically.

37. The method of claim 36, wherein the pharmaceutical composition is administered dermally.

38. The method of claim 23, wherein the pharmaceutical composition is administered topically.

39. The method of claim 38, wherein the pharmaceutical composition is administered dermally.

40. A vector which comprises a sequence encoding the catalytic nucleic acid molecule of claim 1 or 4.

41. A host-vector system comprising a cell having the vector of claim 40 therein.

42. A method of producing the catalytic nucleic acid molecule of claim 1 or 4 comprising culturing a cell having therein a vector comprising a sequence encoding said catalytic nucleic acid molecule under conditions permitting the expression of the catalytic nucleic acid molecule by the cell.

43. A nucleic acid molecule that specifically hybridizes to Hairless Protein mRNA so as to inhibit the translation thereof in a cell.

44. The nucleic acid of claim 43, wherein the nucleic acid is a ribonucleic acid.

45. The nucleic acid of claim 43, wherein the nucleic acid is deoxyribonucleic acid.

46. The nucleic acid molecule of claim 43, wherein the molecule hybridizes to a site within the Hairless Protein mRNA located within the first 3000 residues following the mRNA's 5' terminus.

47. The nucleic acid molecule of claim 46, wherein the

molecule hybridizes to a site within the Hairless Protein mRNA located within the first 1500 residues following the mRNA's 5' terminus.

48. The ribonucleic acid molecule of claim 43, wherein the Hairless Protein mRNA is from a subject selected from the group consisting of human, monkey, rat and mouse.

49. The ribonucleic acid molecule of claim 43, wherein the Hairless Protein mRNA has the sequence shown in Figures 1A - 1C (SEQ ID NO:1).

50. A vector which comprises a sequence encoding the nucleic acid molecule of claim 43.

51. A host-vector system comprising a cell having the vector of claim 50 therein.

52. A pharmaceutical composition comprising (a) the nucleic acid molecule of claim 43 or the vector of claim 50 and (b) a pharmaceutically acceptable carrier.

53. The pharmaceutical composition of claim 52, wherein the carrier is an alcohol.

54. The pharmaceutical composition of claim 53, wherein the carrier is ethylene glycol.

55. The pharmaceutical composition of claim 52, wherein the carrier is a liposome.

56. A method of specifically inhibiting the expression of Hairless Protein in a cell that would otherwise

express Hairless Protein, comprising contacting the cell with the nucleic acid molecule of claim 43 so as to specifically inhibit the expression of Hairless Protein in the cell.

57. A method of specifically inhibiting the expression of Hairless Protein in a subject's cells comprising administering to the subject an amount of the nucleic acid molecule of claim 43 effective to specifically inhibit the expression of Hairless Protein in the subject's cells.

58. A method of specifically inhibiting the expression of Hairless Protein in a subject's cells comprising administering to the subject an amount of the pharmaceutical composition of claim 52 effective to specifically inhibit the expression of Hairless Protein in the subject's cells.

59. A method of inhibiting hair production by a hair-producing cell comprising contacting the cell with an effective amount of the nucleic acid molecule of claim 43.

60. A method of inhibiting hair growth in a subject comprising administering to the subject an effective amount of the pharmaceutical composition of claim 52.

61. A method of inhibiting the transition of a hair follicle from the anagen phase to the catagen phase comprising contacting the follicle with an effective amount of the nucleic acid molecule of claim 43.

62. A method of inhibiting the transition of a hair

follicle from the anagen phase to the catagen phase comprising contacting the follicle with an effective amount of the pharmaceutical composition of claim 52.

63. The method of claim 56, wherein the cell is a keratinocyte.

64. The method of claim 57, wherein the cell is a keratinocyte.

65. The method of claim 58, wherein the cell is a keratinocyte.

66. The method of claim 59, wherein the cell is a keratinocyte.

67. The method of claim 57, wherein the subject is a human.

68. The method of claim 58, wherein the subject is a human.

69. The method of claim 60, wherein the subject is a human.

70. The method of claim 57, wherein the nucleic acid molecule is administered topically.

71. The method of claim 70, wherein the nucleic acid is administered dermally.

72. The method of claim 58, wherein the pharmaceutical composition is administered topically.

73. The method of claim 72, wherein the pharmaceutical composition is administered dermally.

74. The method of claim 60, wherein the pharmaceutical composition is administered topically.

75. The method of claim 74, wherein the pharmaceutical composition is administered dermally.

76. A method of producing the nucleic acid molecule of claim 43 comprising culturing a cell having therein a vector comprising a sequence encoding said nucleic acid molecule under conditions permitting the expression of the nucleic acid molecule by the cell.

77. A non-human transgenic mammal, wherein the mammal's genome:

(a) has stably integrated therein a nucleotide sequence encoding a human Hairless Protein operably linked to a promoter, whereby the nucleotide sequence is expressed; and

(b) lacks an expressible endogenous Hairless Protein-encoding nucleic acid sequence.

FIGURE 1A

tctcccggga gccactcccc tgggcgcctc tccagcccct gggctggaag caccagcaac 60
cctggggatg gggcagaccc tcacagcccg gggtctggag ccggtgtcgg agctcacatg 120
ggcccatgac ctctccagac atttggcaaa atcaaggccc ttagaccagg gacagaccca 180
agcccaggcc ctcccagagg tcataggacg caacccttg tgcccttggg ctatggaaga 240
ggtttgggaa cggctttggg gtggaagatg gccaaaggag cagcttggcc aggtgaggat 300
gaggcagggc agacacaggc cagtggggcg tgccatgtgc cacagatgga gaggaccagg 360
agccagtggc ccggcaggca cagcccggtt ggcgtgggcc agagcgccca tcactgaccc 420
gtgagaactc gactgccct gccagctctg gcactgcccc ctcccagccg ccccgccta 480
gcaccctggg gggcaccccg cccaaccgtg gcctggtccg gcccctcccg ccctttgctc 540
cagttcccgg gcttggcacc tatagtgggg gtgccgcccg cctgccaggc tccggggccg 600
ggcccacggg agggtggggc ggctgggaag ctggcacgct gccccggggg agcctctgtc 660
ggcaggcgcc cgggtgccgc gggggggagg gggaacaaag ggctcattct ccccgtgcgc 720
agccggtggc atcgccgggg cgttggcgga agccccgggg gcccgggagg gggccggccc 780
aggcgcggcc gccgaatcac gggctcctgt ttcccgcagg gtgctggagg aggaaaccgg 840
cggagcagct tccccactct cagttgcgcg tctggcgatg gcgatcagag gtcgtgctgc 900
gctctccgcc gcgctctacc tccattagcc gcgctgcgcg gtgctgcgcc ctcgccggtg 960
cctctctcct gggtcccagg atcggccccc accatccagg cacgaccccc ttccccggcc 1020
cctcggcctt tcccccaact cggccatctc cgacccgggg cgcgtgttcc ccccggcccg 1080
gcgccttctc tccctccggg ggcacccgct ccctagcccc ggcccggccc tccccgcggc 1140
gcagcacgga gtctcggcgt cccatggcgc aacctacggc ctcggcccag aagctggtgc 1200
ggccgatccg cgccgtgtgc cgcatcctgc agatcccgga gtccgacccc tccaacctgc 1260
ggccctagag cgcccccgcc gccccggggg aaggagagcg cgagcgcgct gagcagacag 1320
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aggcgcccgg tgccggccac gacgaccgcc accgcccgcg ccgcgaccgg ccggtgaagc 1440
ccagggaccc ccctctggga gagccccatg agggcaggag agtgatggag agtacgccca 1500
gcttcctgaa gggcacccca acctgggaga agacggcccc agagaacggc atcgtgagac 1560
aggagcccgg cagcccgcct cgagatggac tgcaccatgg gccgctgtgc ctgggagagc 1620
ctgctccctt ttggaggggc gtcctgagca ccccagactc ctggcttccc cctggcttcc 1680
cccagggccc caaggacatg ctcccacttg tggagggcga gggcccccag aatggggaga 1740
ggaaggtcaa ctggctgggc agcaaagagg gactgcgctg gaaggaggcc atgcttaccc 1800
atccgctggc attctgcggg ccagcgtgcc cacctcgctg tggcccccctg atgcctgagc 1860
atagtggtgg ccatctcaag agtgaccctg tggccttccg gccctggcac tgccctttcc 1920

FIGURE 1B

ttctggagac caagatcctg gagcgagctc ccttctgggt gcccacctgc ttgccaccct 1980
acctagtgtc tggcctgccc ccagagcatc catgtgactg gcccctgacc ccgcacccct 2040
gggtatactc cgggggccag cccaaagtgc cctctgcctt cagcttaggc agcaagggct 2100
tttactacaa ggatccgagc attcccaggt tggcaaagga gcccttggca gctgcggaac 2160
ctgggttgtt tggcttaaac tctggtgggc acctgcagag agccggggag gccgaacgcc 2220
cttcactgca ccagagggat ggagagatgg gagctggccg gcagcagaat ccttgcccgc 2280
tcttcctggg gcagccagac actgtgccct ggacctcctg gcccgcttgt cccccaggcc 2340
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tggggccacc agcaacacca aggtgccccc ctcctgagcc gcctgtcacc cagcggggct 2460
gctgttcatc ctacccaccc actaaaggtg gggatcttgg cccttgtggg aagtgccagg 2520
agggcctgga ggggggtgcc agtggagcca gcgaacccag cgaggaagtg aacaaggcct 2580
ctggccccag ggcctgtccc cccagccacc acaccaagct gaagaagaca tggctcacac 2640
ggcactcgga gcagtttgaa tgtccacgcg gctgccctga ggtcgaggag aggccggttg 2700
ctcggctccg ggcccctcaaa agggcaggca gccccgaggt ccagggagca atgggcagtc 2760
cagcccccaa gcggccaccg gacccttcc caggcactgc agaacagggg gctgggggtt 2820
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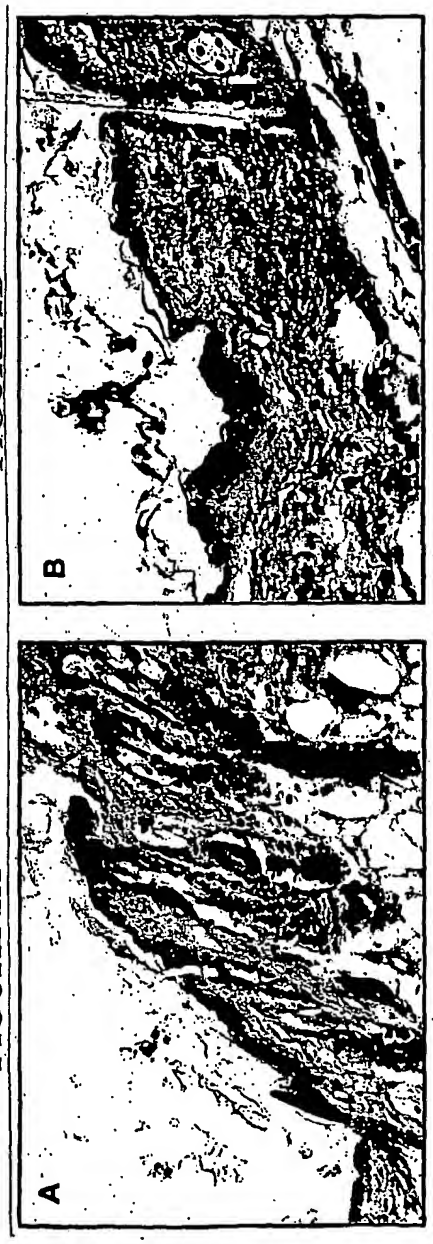


FIGURE 2A

FIGURE 2B

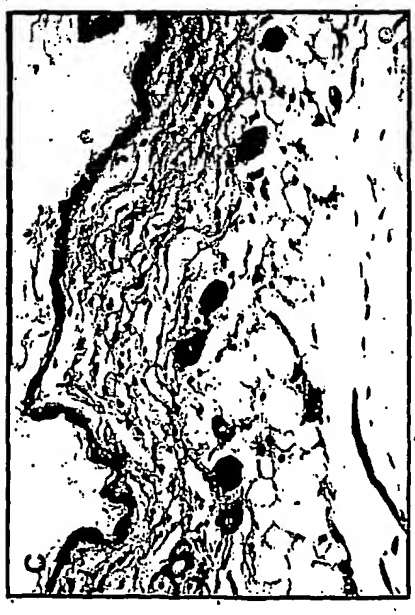


FIGURE 2C

FIGURE 2D

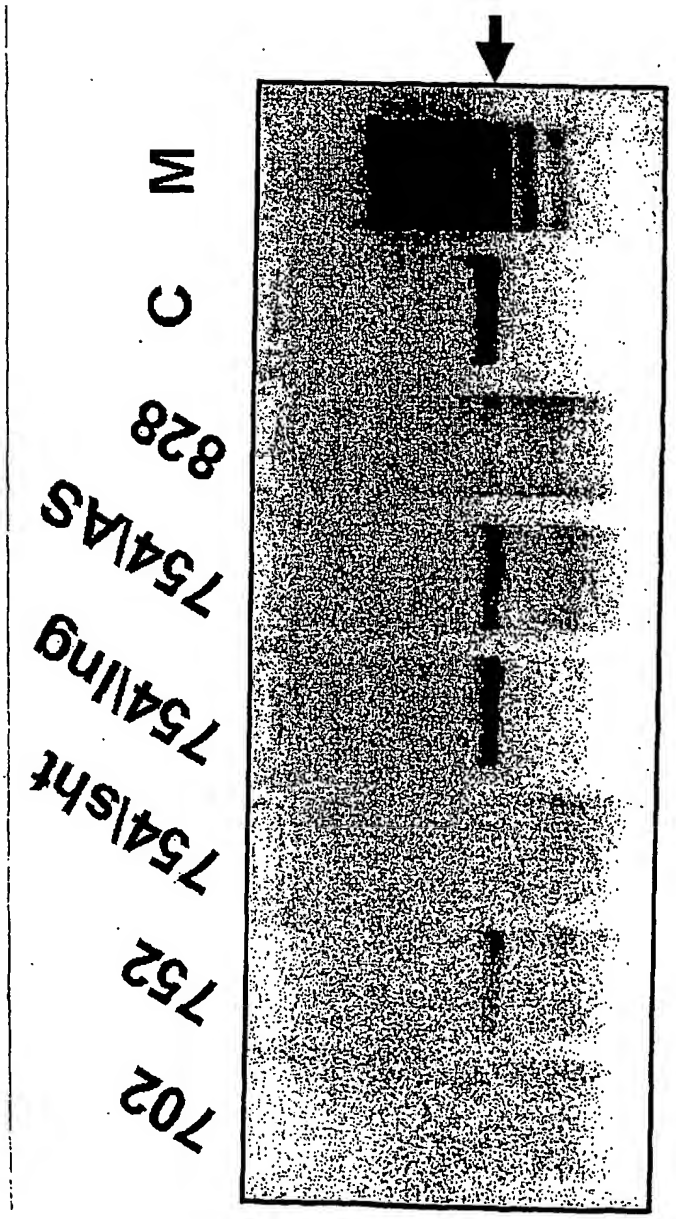
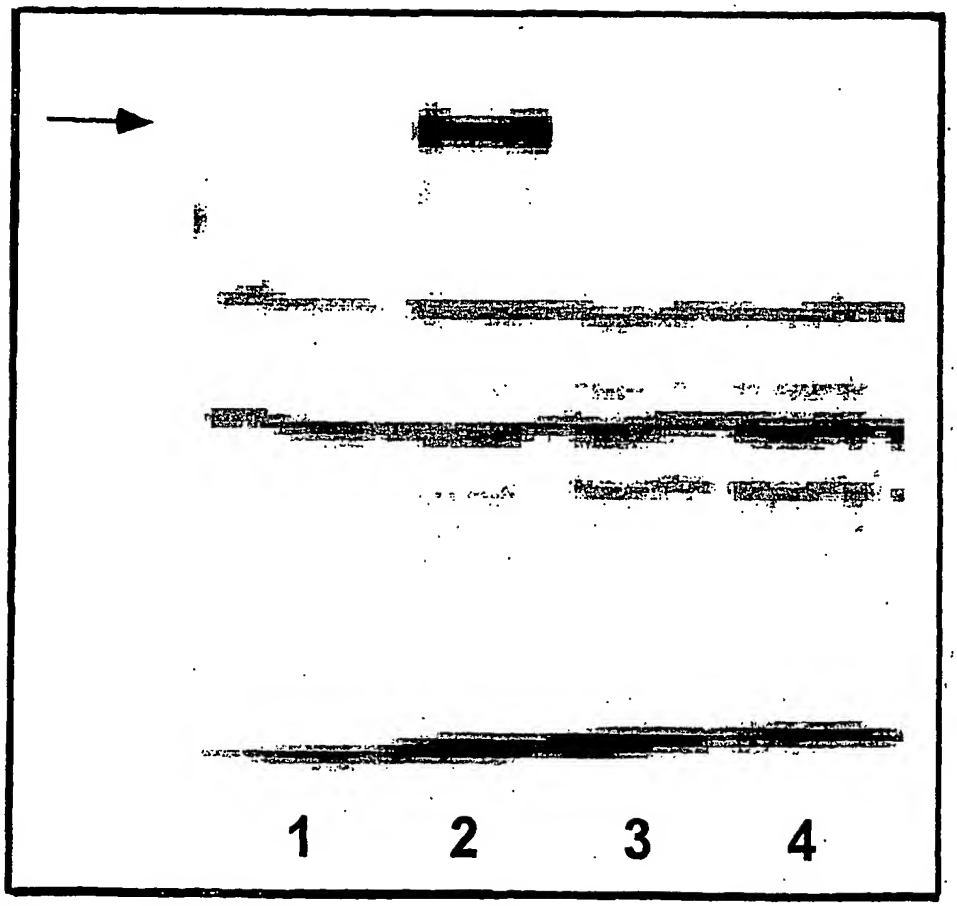


FIGURE 3

FIGURE 4



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Gly Leu Gly Asp Arg Leu Cys Arg Leu Leu Arg Arg Glu Arg Glu Ala
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Leu Ala Trp Ala Gln Arg Glu Ser Gln Gly Pro Ala Val Thr Glu Asp
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Ser Pro Gly Ile Pro Arg Cys Cys Ser Arg Cys His His Gly Leu Phe
        595                 600                 605

Asn Thr His Trp Arg Cys Pro Arg Cys Ser His Arg Leu Cys Val Ala
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Cys Gly Arg Val Ala Gly Thr Gly Arg Ala Arg Glu Lys Ala Gly Phe
625                 630                 635                 640

Gln Glu Gln Ser Ala Glu Glu Cys Thr Gln Glu Ala Gly His Ala Ala
                645                 650                 655

Cys Ser Leu Met Leu Thr Gln Phe Val Ser Ser Gln Ala Leu Ala Glu
            660                 665                 670

Leu Ser Thr Ala Met His Gln Val Trp Val Lys Phe Asp Ile Arg Gly
        675                 680                 685

His Cys Pro Cys Gln Ala Asp Ala Arg Val Trp Ala Pro Gly Asp Ala
    690                 695                 700

Gly Gln Gln Lys Glu Ser Thr Gln Lys Thr Pro Pro Thr Pro Gln Pro
705                 710                 715                 720

Ser Cys Asn Gly Asp Thr His Arg Thr Lys Ser Ile Lys Glu Glu Thr
                725                 730                 735

Pro Asp Ser Ala Glu Thr Pro Ala Glu Asp Arg Ala Gly Arg Gly Pro
            740                 745                 750

Leu Pro Cys Pro Ser Leu Cys Glu Leu Leu Ala Ser Thr Ala Val Lys
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Leu Cys Leu Gly His Glu Arg Ile His Met Ala Phe Ala Pro Val Thr
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Pro Ala Leu Pro Ser Asp Asp Arg Ile Thr Asn Ile Leu Asp Ser Ile
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Ile Ala Gln Val Val Glu Arg Lys Ile Gln Glu Lys Ala Leu Gly Pro
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Gly Leu Arg Ala Gly Pro Gly Leu Arg Lys Gly Leu Gly Leu Pro Leu
            820                 825                 830

Ser Pro Val Arg Pro Arg Leu Pro Pro Pro Gly Ala Leu Leu Trp Leu
        835                 840                 845

Gln Glu Pro Gln Pro Cys Pro Arg Arg Gly Phe His Leu Phe Gln Glu
    850                 855                 860

His Trp Arg Gln Gly Gln Pro Val Leu Val Ser Gly Ile Gln Arg Thr
865                 870                 875                 880

Leu Gln Gly Asn Leu Trp Gly Thr Glu Ala Leu Gly Ala Leu Gly Gly
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Gln Val Gln Ala Leu Ser Pro Leu Gly Pro Pro Gln Pro Ser Ser Leu
            900                 905                 910

Gly Ser Thr Thr Phe Trp Glu Gly Phe Ser Trp Pro Glu Leu Arg Pro
        915                 920                 925

Lys Ser Asp Glu Gly Ser Val Leu Leu Leu His Arg Ala Leu Gly Asp
    930                 935                 940

Glu Asp Thr Ser Arg Val Glu Asn Leu Ala Ala Ser Leu Pro Leu Pro
945                 950                 955                 960

Glu Tyr Cys Ala Leu His Gly Lys Leu Asn Leu Ala Ser Tyr Leu Pro
                965                 970                 975

Pro Gly Leu Ala Leu Arg Pro Leu Glu Pro Gln Leu Trp Ala Ala Tyr
            980                 985                 990

Gly Val Ser Pro His Arg Gly His  Leu Gly Thr Lys Asn  Leu Cys Val
        995                 1000                1005

Glu Val  Ala Asp Leu Val Ser  Ile Leu Val His Ala  Asp Thr Pro
    1010                1015                1020

Leu Pro  Ala Trp His Arg Ala  Gln Lys Asp Phe Leu  Ser Gly Leu
    1025                1030                1035

Asp Gly  Glu Gly Leu Trp Ser  Pro Gly Ser Gln Val  Ser Thr Val
    1040                1045                1050

Trp His  Val Phe Arg Ala Gln  Asp Ala Gln Arg Ile  Arg Arg Phe
    1055                1060                1065

Leu Gln  Met Val Cys Pro Ala  Gly Ala Gly Ala Leu  Glu Pro Gly
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Ala Pro  Gly Ser Cys Tyr Leu  Asp Ala Gly Leu Arg  Arg Arg Leu
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    1085                1090                1095

Arg Glu  Glu Trp Gly Val Ser  Cys Trp Thr Leu Leu  Gln Ala Pro
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Gly Glu  Ala Val Leu Val Pro  Ala Gly Ala Pro His  Gln Val Gln
    1115                1120                1125

Gly Leu  Val Ser Thr Val Ser  Val Thr Gln His Phe  Leu Ser Pro
    1130                1135                1140

Glu Thr  Ser Ala Leu Ser Ala  Gln Leu Cys His Gln  Gly Pro Ser
    1145                1150                1155

Leu Pro  Pro Asp Cys His Leu  Leu Tyr Ala Gln Met  Asp Trp Ala
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Val Phe  Gln Ala Val Lys Val  Ala Val Gly Thr Leu  Gln Glu Ala
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Lys
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